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Mammalian mitochondria contain a soluble acyl carrier protein

John E. Cronan*, Ian M. Fearnley, John E. Walker

Medical Research Council Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2XY, United Kingdom

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Abstract Plant and fungal mitochondria contain type II fatty acid synthesis systems closely related to those of bacteria in which the individual reactions are catalyzed by separate soluble proteins acting on intermediates bound to acyl carrier protein (ACP). Mammalian mitochondria are thought to synthesize fatty acids, but evidence for the key ACP component was lacking since the only reported ACP was the SDAP subunit of the membrane-bound NADH:ubiquinone oxidoreductase, We report that most of the SDAP is found in the soluble (matrix) fraction of bovine heart mitochondria and is therefore available to carry the intermediates of type II fatty acid synthesis.

Keywords: Acyl carrier protein; Mitochondrial matrix; Type II fatty acid synthesis; NADH:ubiquinone oxidoreductase; Complex I

1. Introduction

Fatty acid synthesis in eukaryotes is catalyzed by three distinct enzyme systems. The great bulk of the fatty acid moieties of the membrane lipids are made by the cytosolic type I fatty acid synthase (FAS), a very large multifunctional protein that produces acyl chains of 16–18 carbon atoms. These chains can then be further elongated by the microsomal fatty acid elongases to give the very long chain sphingolipid fatty acids. Mitochondrial fatty acid synthesis seems derived from the progenitor bacterium because it is of the type II class in that a discrete soluble protein catalyzes each step of the reaction cycle.

The evidence for a mitochondrial type II fatty acid synthesis system has markedly strengthened in the past few years [1–4] and is strongest in the yeast, *Saccharomyces cereviseae*. The yeast genome encodes mitochondrially-localized proteins that comprise an essentially complete set of type II fatty acid synthesis proteins. Moreover, enzymatic activity and mitochondrial localization have been demonstrated for several of the yeast proteins (see [1,2] and references therein). Deletion of any of the genes encoding these proteins gives a consistent phenotype; the deletion mutants fail to grow on non-fermentable carbon sources and the cellular levels of lipoic acid are very low [1,5–8]. These phenotypes indicate that the primary role of the mitochondrial fatty acid synthesis system is assembly of the octanoate precursor of lipoic acid, an essential cofactor of aerobic metabolism [1].

Studies in *Escherichia coli* indicate that lipoic acid synthesis requires two proteins, LipA and LipB [9]. LipA is responsible for insertion of the sulfur atoms at C6 and C8 to give the reduced dithiol form of lipoic acid [10,11]. However, the substrate of LipA is not free lipoic acid, but rather octanoic acid that is amide-linked to an enzyme protein, such as pyruvate dehydrogenase, that requires lipoic acid for function. That is, lipoic acid is assembled on its cognate proteins [9,11]. The function of LipB is to catalyze formation of the octanoyl-protein amide linkage to provide substrates for LipA [9]. Homologues of LipA and LipB are found in bacteria, fungi, plants, and animals and thus the *E. coli* pathway seems likely to be general. Indeed, the human and plant LipAs [8,12] and the plant LipB [13] have been shown to complement growth of *E. coli lipA* and *lipB* mutants, respectively. Moreover, LipB mutants of *S. cereviseae* [14] and of another yeast, *Kluyveromyces lactis* [15] are deficient in lipoic acid-dependent enzyme activities and isolated plant mitochondria synthesize large amounts of the octanoylated form of a lipoic acid-modified protein [4]. However, in mammalian mitochondria evidence for a type II fatty acid synthesis system is problematic. The functions of only five of the needed mitochondrial proteins have been demonstrated in vitro [3,16,17] and two of these are the key acyl carrier protein (ACP) component and the enzyme that attaches the essential 4′-phosphopantotheine (4′-PP) prosthetic group to ACP. Interpretation of these data is severely complicated by the fact that the ACP tested in vitro has only been observed in an insoluble form in vivo [18–22], whereas the known type II fatty acid synthesis proteins are all soluble. Specifically, the ACP was the ACP-like mitochondrial protein, SDAP subunit (named for its N-terminal sequence), of the firmly membrane bound NADH:ubiquinone oxidoreductase (complex I) of mitochondrial respiratory chain [18,19]. Complex I has a molecular mass of approximately 900 kDa and is composed of 46 different proteins, most of which (like SDAP) are encoded by the nuclear genome [19].

These considerations raised the question of whether or not complex I-containing mitochondria (*S. cereviseae* lacks complex I) also contain a soluble ACP available for fatty acid synthesis. Complex I-containing organisms might contain two distinct ACP species, one targeted to complex I and another

*Corresponding author. Permanent address: Department of Microbiology, University of Illinois, B103 Chemical and Life Sciences Laboratory, 601 S. Goodwin Avenue, Urbana, IL 61801, USA. Fax: +1 217 244 6697.
E-mail address: j-cronan@life.uiuc.edu (J.E. Cronan).

Abbreviations: ACP, acyl carrier protein; SDAP; ACP-like mitochondrial protein (named for its N-terminal sequence); 4′-PP, 4′-phosphopantetheine

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targeted to the matrix. Indeed, *Drosophila melanogaster* has two ACP forms generated by alternative splicing that differ their N-terminal sequences [23] whereas the *Caenorhabditis elegans* expresses three putative mitochondrial ACPs [www.wormbase.org]. The expressed sequence tag database of the human and mouse genomes show only mRNAs encoding homologues of the SDAP protein. Although there are translated genomic sequences that align partially with ACPs, there is no evidence that these sequences are expressed or, if expressed, that the spliced mRNAs encode mitochondrial targeted ACPs. For these reasons we assayed the matrix fraction of bovine heart mitochondria for proteins that were both very small and very acidic, these being the most general attributes of ACP-like proteins. The only ACP-like matrix protein we obtained from bovine heart mitochondria was SDAP, the protein previously isolated from complex I. However, we report that the bulk of the mitochondrial SDAP protein is soluble and thus proficient to participate in type II fatty acid synthesis.

2. Materials and methods

2.1. Purification of the matrix SDAP

The mitochondrial matrix fractions were obtained as byproducts of complex I purifications done in a neighboring laboratory [20,21,24]. In the first step of these purifications frozen packed mitochondria equivalent to 2 bovine hearts were thawed overnight at 4 °C. The thawed mitochondria were suspended in 2 L of 4 °C water and blended for two min (medium speed) in a Waring blender. KCl (22.3 g, 0.15 M final concentration) was added followed by 3 min of further blending. The suspension was centrifuged at $8600 \times g$ for 40 min and the resulting supernatant was removed to fresh bottles and the centrifugation repeated to give the low-speed supernatant. This, in turn, was centrifuged at $220\,000 \times g$ for 90 min to give the high-speed supernatant (matrix) and a high-speed membrane pellet. One-twentieth volume of 0.5 M sodium-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 6.1 was added to the matrix fraction (final pH was 6.2–6.4) and the buffered matrix was fractionated by ion exchange chromatography on either DEAE or Q resins. However, chromatography was often confounded by the slow formation of a fine non-protein precipitate that clogged the columns during the loading process. Repeating the high-speed centrifugation removed the precipitate, but it reformed. For this reason fractionation was done on Vivapure D-H or Q-H centrifugal ion exchange spin columns that allowed rapid and quantitative loading of the acidic proteins before the fine precipitate could form. The fractionation protocol followed the manufacturer recommendations except that the eluting salt was LiCl and the columns were equilibrated by washing with a 10-fold concentrate of the loading buffer followed by washing with loading buffer (the loading and elution buffers were 25–50 mM sodium HEPES, pH 6.1–6.2). Purification was followed by native gel electrophoresis on 10–20% Tris glycine gels (Invitrogen) followed by staining with Coomassie Blue.

2.2. Purification of membrane-bound SDAP

Membrane pellets were dissolved in 8 M guanidine HCl then this was removed by extensive dialysis against the loading buffer. The copious protein precipitate was removed by centrifugation and the supernatant was fractionated identically to the matrix. The membrane-derived SDAP behaved identically to the soluble SDAP on the ion exchange spin columns.

2.3. Mass spectrometry

Protein molecular masses were measured using a triple quadrupole mass spectrometer with electrospray ionization in positive ion mode (either a Sciex API III+ or a Micromass Quattro Ultima instrument). Protein samples were dialysed against 50 mM ammonium bicarbonate then diluted and acidified with a solution of 1% formic acid in 50% aqueous acetonitrile. The acidified samples were introduced to the spectrometer by flow injection at 3 μl per min in a stream of 50% aqueous acetonitrile. Electrospray mass spectra were recorded in mass ranges between 900 and 2100 or 700 and 1700 mass to charge ratios.

3. Results

3.1. The mammalian mitochondrial matrix contains a soluble ACP

ACPs are unusual proteins in that they are of very small size (<80 residues) and are highly acidic (pI 3.9–4.3). Therefore, we enriched for ACPs by isolation of the most acidic proteins of the mitochondrial matrix by ion exchange chromatography. It should be noted that the matrix fractions analyzed were obtained using exactly the same lysis procedure used to obtain the mitochondrial membranes from which the original [18] and all subsequent [20–22,24–26] complex I preparations in which SDAP has been demonstrated. Indeed, other investigators subsequently isolated SDAP-containing complex I from the membranes of the same lots of mitochondria from which the matrix fractions we analyzed were derived. Given this history and the fact that the association of SDAP with the other complex I subunits survives many treatments including splitting of the complex into two protein fractions by detergent treatment [20,22,26], it seems very unlikely that any matrix SDAP was released from the complex by the mild procedure used to lyze the mitochondria.

The ion exchange columns were loaded at pH 6.1–6.2 in the presence of 150 mM KCl to ensure that only highly acidic proteins such as ACPs would bind. Following elution with increasing LiCl concentrations we readily found a pair of proteins that bound tightly to the ion exchange resin and migrated just behind *E. coli* ACP on native gel electrophoresis using tightly cross-linked gels (Fig. 1). Their rapid electrophoretic mobilities on these gels indicated that these proteins were both small and highly acidic (Fig. 1). The two stained protein bands were then cut from the gel, soaked in water and placed in the wells of a second gel that was run in the presence of 0.1% sodium dodecyl sulfate. In this method electrophoresis acts to destain, elute and denature the protein(s) of the gel segments [18]. The proteins of the second gel were then transferred to an Immobilon P membrane and the stained bands were submitted to N-terminal sequencing. The fastest migrating band from an early matrix preparation gave the sequence SDAPPLTLEGIKDRVLYVLK and a preparation made several months later using mitochondria from a different pool of hearts gave the same sequence for the first eight cycles of Edman degradation. Both sequences precisely matched that reported for the SDAP subunit of complex I [14] indicating that the mitochondrial matrix contained soluble SDAP. The soluble SDAP migrated slightly behind *E. coli* ACP on native gel electrophoresis consistent with its greater size (10 109 vs. 8502 Da), but very similar pI (calculated as pH 4.2). The band migrating behind SDAP had the N-terminal sequence SVRKFTEKHEXVTTE (a later preparation gave SVRKFTEK) which identified the band as the H protein subunit of the glycine cleavage enzyme, a well characterized mitochondrial matrix protein having a molecular weight of 13 846 Da and a calculated pI of 4.4 [19]. In some matrix preparations a third band was seen that migrated immediately behind the H protein band and had the same amino terminal sequence. However, the relative

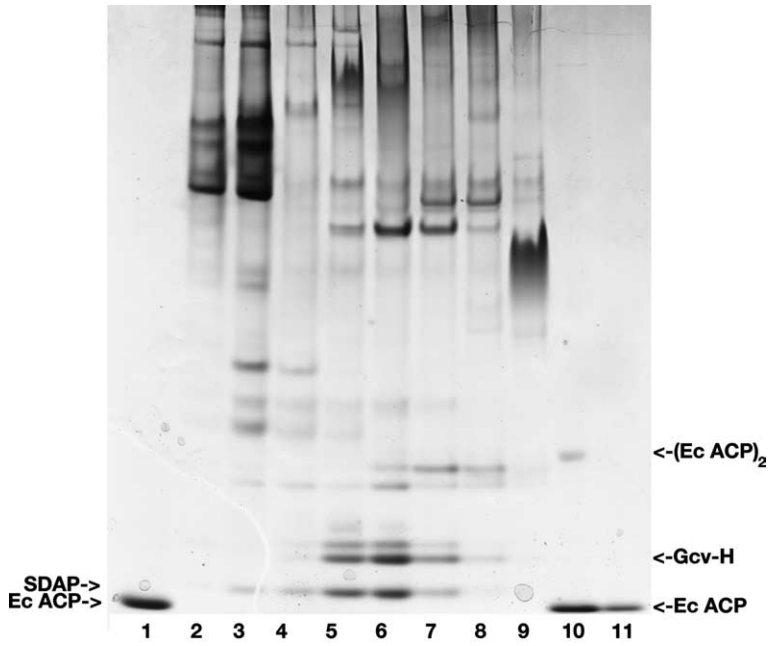

Fig. 1. Native gel electrophoresis of the acidic proteins of bovine heart mitochondrial matrix. Fractionation of the high speed supernatant on a DEAE membrane is shown. Lanes 1 and 11, *E. coli* ACP (Ec Acp) freshly reduced with DTT. Lanes 2–9 are fractions of the DEAE membrane-bound proteins eluted by increasing concentrations of LiCl. The LiCl concentrations in lanes 2–9 were 0.25, 0.30, 0,35, 0.40. 0.45, 0.50, 0.60, and 1.0 M, respectively. In lane 10, the ACP had not been freshly reduced and some disulfide dimer was present. Symbols: Gcv-H, glycine cleavage enzyme H subunit.

proportions of the second and third bands varied among preparations suggesting some preparation-dependent modification of H protein. The SDAP and the H proteins were reproducibly detected in all ten matrix fractions obtained from mitochondria prepared from four different pools of hearts.

3.2. Interrelationship of the soluble and membrane-bound forms of SDAP

The first question to be answered was whether or not the form of SDAP found in the matrix fraction was a discrete soluble protein rather than a subassembly of complex I. The matrix SDAP present in the high speed supernatant passed through a protein concentration cartridge (Millipore Biomax) having a nominal molecular weight cutoff of 50 000 and also passed through dialysis tubing having a similar cutoff. In both cases the SDAP was accompanied by two known soluble proteins, the H protein and cytochrome *c* (12.3 kDa, released by disruption of the mitochondrial outer membrane). Like *E. coli* ACP the matrix SDAP was soluble in 50% 2-propanol, readily renatured from trichloroacetic acid precipitates and had an ion exchange chromatographic behavior that was virtually identical to that of the *E. coli* protein. Both properties are inconsistent with SDAP being bound to other proteins. Moreover, the ion exchange chromatographic behavior of SDAP was unaltered by trichloroacetic acid treatment. Finally, SDAP purified by ion exchange chromatography was found to migrate in size exclusion chromatography as a protein of 22 kDa (Fig. 2). As expected from the gel electrophoresis results the size exclusion chromatographic behavior of SDAP was virtually identical to that of *E. coli* ACP, a protein having an unusually large Stokes radius that accounts for its aberrant behavior in hydrodynamic analyses [27]. From these data we conclude that the matrix SDAP is a discrete soluble protein.

A remaining question was whether or not the soluble SDAP could somehow be derived from traces of membrane fragments remaining in the high-speed supernatants. Therefore we used guanidine-HCl to dissolve the total membrane pellets separated from the matrix fraction by ultracentrifugation. After removal of the denaturant by dialysis the proteins that remained soluble were fractionated in parallel with the matrix proteins by ion exchange chromatography. The only protein band reproducibly found in both the matrix and membrane fractions was SDAP (Fig. 3). The total membrane bound SDAP found in the high speed pellets of the various matrix preparations analyzed was at most about 20% of the total SDAP and generally $\ll$10%. Therefore, even if SDAP had somehow become solubilized from any membrane fragments that might have remained in the matrix fraction, this could not have provided significant levels of the protein. Finally, we measured the relative levels of SDAP in the soluble and membrane-bound forms (Fig. 3). The major form was soluble SDAP and comprised about 70% of the total mitochondrial SDAP. The amount of SDAP was estimated by densitometry of Coomassie blue stained gels using *E. coli* ACP was as a standard because small acidic proteins bind significantly less of this stain than do proteins of more usual amino acid composition. Given this standardization the mitochondria of two ox hearts contained about 15 nmol of SDAP. This would comprise roughly 0.02% of the total matrix protein.

3.3. Forms of the matrix SDAP

The complex I SDAP was shown to be modified by attachment of 4′-PP [18] and this is also true of the soluble SDAP in that a species of 10447.5 was detected by electrospray mass spectrom-

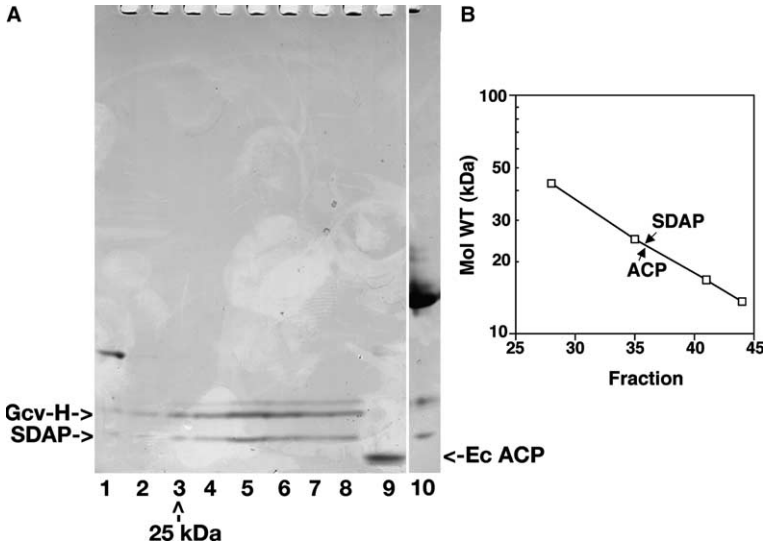

Fig. 2. Size exclusion chromatography of matrix SDAP. Lanes 1–8 are fractions 32–39 from the included volume of the column. The peak fraction of the elution profile of α-chymotrypsinogen is shown by the arrow. For comparison *E. coli* ACP (lane 9) and the peak fraction of guanidine-solubilized SDAP from the high-speed membrane pellet of the same matrix preparation are shown (lane 10). The membrane-bound SDAP prepared from the pooled high-speed pellet is compared to 25% of the matrix preparation. Both forms of SDAP were purified by ion exchange chromatography then concentrated and desalted by ultrafiltration before loading on a 1.5×46 cm Sephadex G75 (Superfine) column eluted in loading buffer at 0.175 ml/min with 0.4 ml fractions. The molecular weight standards were ovalbumin, α-chymotrypsinogen, myoglobin, and RNAase A (molecular weights of 43, 25, 16.9, and 13.7 kDa, respectively).

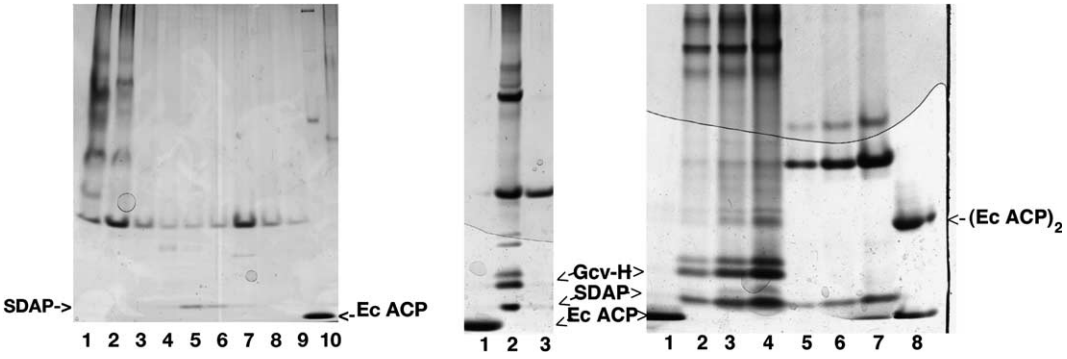

Fig. 3. Membrane-bound and soluble SDAP. Panel A. Ion exchange purification of the soluble proteins obtained by guanidine-extraction of membranes followed by dialysis. Lanes 1–9 are successive elutions of proteins from the DEAE membrane by increasing concentrations of LiCl. The LiCl concentrations in lanes 1–9 were 0.15, 0.20, 0.25, 0.30, 0.35, 0.40. 0.45, 0.50, 0.60, and 1.0 M, respectively. Lane 10 is freshly reduced *E. coli* ACP. Panel B shows pooled SDAP-containing ion exchange fractions from either the matrix or high-speed pellet of the same matrix preparation. The samples loaded on lanes 2 and 3 were equivalent to one-hundredth of the soluble SDAP and high-speed pellet SDAP, respectively. Lane 1 is freshly reduced *E. coli* ACP. Densitometry of lanes 2 and 3 indicated at the SDAP concentration of lane 3 was 11% that of lane 2. Panel C shows pooled SDAP-containing ion exchange fractions from either the matrix or total membrane (low-speed plus high-speed pellets) of the same matrix preparation. Lanes 2–4 were loaded with increasing volumes (5, 10, and 20 μl, respectively) of the soluble SDAP fraction whereas lanes 5–7 were loaded with increasing volumes (5, 10, and 20 μl, respectively) of the total membrane SDAP fraction. Densitometry of lanes 2, 3, 4 and 6 indicated that 70% of the total SDAP is soluble.

etry (4′-PP-modified SDAP has a calculated mass of 10448.9). In a different matrix SDAP preparation a major species of mass 10577.2 was detected. This is the mass of 4′-PP-modified SDAP that has been further modified by attachment of octanoic acid (calculated mass of 10575.0) to the 4′-PP thiol which is a putative precursor of lipoic acid. We have not detected the disulfide-linked dimeric form of SDAP by gel electrophoresis even following treatment with the oxidizing agents, potassium ferricyanide and oxidized dithiothreitol. In contrast, *E. coli* ACP readily forms disulfide-linked dimers even without intentional oxidation. The difference between ACP and SDAP could be due to blocking of the SDAP 4′-PP thiol by acylation as in the case of octanoyl-SDAP, but this cannot be the sole explanation since a non-acylated form of SDAP was also detected. It seems more likely that the lack of disulfide-linked dimer formation is due to formation of intramolecular mixed disulfides between the 4′-PP thiol and that of the Cys-72 side chain. Indeed, modeling of SDAP using the crystal structure of *E. coli* butyryl-ACP [28] suggests that formation of the intramolecular disulfide would proceed readily.

4. Discussion

Our finding that SDAP is present in bovine heart mitochondria as a soluble matrix protein in addition to the form found

in the inner membrane complex I form greatly strengthens the proposal of Smith and coworkers [3] that ''there is a malonyl-CoA/ACP-dependent FAS system that functions inside mammalian mitochondria''. It seems likely that during the assembly of complex I, the charge and/or structure of SDAP results in recruitment of the protein into the complex. The SDAP of complex I is 4′-PP-modified and the 4′-PP thiol is acylated [18,19] indicating prior interactions with the soluble 4′-PP transferase and type II fatty acid synthase proteins prior to incorporation into the complex (although unprecedented, it remains possible that complex I-bound SDAP could participate in these reactions). Note that *E. coli* ACP has recently been shown to bind proteins other than those involved in lipid synthesis [29]. Thus, ACP, like SDAP, may play roles in diverse cellular processes.

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